REMARKS

I. Status of Claims

Claims 1-46 were filed with the application. Claims 25-46 stand withdrawn pursuant to a restriction requirement and are canceled here. Thus, claims 1-24 remain pending, have been examined, and stand rejected, variously, under 35 U.S.C. §112, second paragraph, 35 U.S.C. §112, first paragraph, 35 U.S.C. §102, and 35 U.S.C. §103. The specific grounds for rejection, and applicants' response thereto, are set out in detail below.

II. Sequence Compliance

The examiner has identified sequences at page 48 (lines 12-13) and page 51 (lines 28-29) that do not have corresponding SEQ ID NOs associated with them. An amendment and substitute sequence listing are provided herewith correcting the deficiency.

III. Oath and Declaration

The oath/declaration is said to be defective in lacking a ***dated*** signature for Gregory Evans. Applicants are in the process of obtaining a replacement oath/declaration, which will be submitted shortly.

IV. Objections

Claim 5 is objected to because of a misspelling. Claim 14 is objected to due to a grammatical errors. Both have been corrected by amendments. Reconsideration and withdrawal of the objections is therefore respectfully requested.

V. Rejections Under 35 U.S.C. §112, Second Paragraph

Claims 2, 9, 11, 12 and 19-24 stand rejected under the second paragraph of §112, as indefinite. The individual rejections are addressed below:

Claim 2: The examiner argues that the citation of a series of cells does not indicate whether all of these cells are part of the same conduit, or used individually. An amendment replacing "and" with "or" has been provided. However, given that claims 1 and 2 use the term "comprising," the use of "or" does not preclude the presence of other cell types.

Claims 9, 11 and 12: The examiner argues that the use of the term "gene" is inconsistent with the subsequent recitation of a gene product, *i.e.*, a polypeptide. A clarifying amendment is provided.

Claims 19-24: The term "induction" in claims 19-24[1] is said to lack antecedent basis. Amendments have been provided to address the rejection.

In light of the preceding, applicants believe that each ground of rejection has been addressed. Therefore, reconsideration and withdrawal of the rejections is respectfully requested.

VI. Rejection Under 35 U.S.C. §112, First Paragraph (Written Description)

Claims 8-14 are rejected under the first paragraph of §112 as allegedly lacking a legally sufficient written description. According to the examiner, the specification is deficient in not describing "cell kill genes" given the unpredictability in the art and the lack of a correlation between structure and function. Applicants traverse.

[1] Claim 19 does not contain the limitation "induction." Thus, rejection of this claim is believed to be an error.

The examiner has not stated any rational basis for challenging the generic disclosure of "cell kill genes." First and foremost, there is no "structure-function" relationship between various cell kill genes given that they are quite distinct in how they accomplish their role in killing of cells. Thus, it is non-sensical in this context to suggest that such is required. Moreover, it also makes no sense to require a detailed discussion of genomic clones, including introns and regulatory sequences, since there is nothing in the specification, and nothing provided in the Office Action, to indicate that these elements are in any way required for the present invention. Finally, the examiner's comments on "herbicide tolerance genes" are not understood.

The only relevant issue raised by the examiner is whether "cell kill genes" as a genus were known at the time of filing. Applicants submit that the specification, which identifies (a) regulated expression of toxins and (b) enzyme/prodrug combinations, shows that cell kill genes clearly were known. The specific example of HSV-tk is provided for the latter type of cell kill gene. See Specification, paragraph bridging pages 4-5. As such, there is little questions that a genus of "cell kill genes" was supported at the time of filing.

In light of the foregoing, applicants respectfully request reconsideration and withdrawal of the rejection.

VII. Rejection Under 35 U.S.C. §112, First Paragraph (Enablement)

Claims 1-33 are rejected under the first paragraph of §112 as allegedly lacking an enabling disclosure.[2] The examiner has provided an analysis of five different Wands factors; as such, the analysis is incomplete. Moreover, the examiner has cited no evidence to support

[2] Claims 25-33 have been withdrawn and are canceled herein. It is believed that their inclusion in this rejection was in error.

factual assertions that (a) the state of the art is devoid of clinical success, (b) that there is a "lack of methods steps for use of the [present] invention," (c) that the art of nerve repair is unpredictable, (d) that there is a lack of correlation between the experimental model used and the claimed invention. Thus, in essence, the examiner simply questions whether or not the claimed invention will work without any supporting evidence.

It is black letter law that the Patent Office must take appellants' specification as in compliance with enablement requirements unless there is reason to doubt "the objective truth" of the specification. Otherwise, there would be no need for appellants to go to the trouble and expense of supporting a "presumptively accurate disclosure." *In re Marzocchi*, 169 UPSQ 370 (CCPA 1971). The examiner further is requested to provide an affidavit under 37 C.F.R. §1.104(d)(2) as the rejection appears to be based solely on "facts within the personal knowledge of an employee of the Office." If no supporting evidence or affidavit is provided, it is requested that the rejection be withdrawn.

Furthermore, as described in the attached declaration under 37 C.F.R. §1.132 from Gregory R.D. Evans, the inventors have successfully identified suitable conduit material and created helper cells transformed with an inducible promoter that directs expression of a growth factor.[3] Thus, the critical steps in preparing materials for use in the claimed method have been accomplished, and there is no reason to doubt that such materials cannot be placed into a suitable animal. This is further evidence of enablement for the present invention.

In light of the foregoing, applicants respectfully submit that a *prima facie* case of non-enablement has not been established, but were such a case present, the evidence provided rebut even a valid *prima facie* rejection. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

VIII. Rejection Under 35 U.S.C. §102

Claims 1-7 are said to be anticipated by Hadlock *et al.* The reference is said to teach a neural regeneration conduit that is porous and can be comprised of PLGAS or PLLA. The conduit is said to include a layer of cells such as Schwann cells that can be engineered for the overexpression of neurotropic factors or NGF through recombinant expression. The conduits are said to be implanted into a subject adjacent to nerve tissues.

Claim 1 has now been amended to incorporate the elements of previous claim 4, namely, inducible expression of growth factors. The examiner has not pointed to any such disclosure in Hadlock, nor have applicants been able to identify any. As such, applicants believe the rejection of previous claim 4, and now claim 1, over Hadlock is improper. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

IX. Rejection Under 35 U.S.C. §103

Claims 1-7 and 15-18 are said to be rendered obvious by the combination of Hadlock in view of U.S. Patent 5,888,774 ("the '774 patent"). Hadlock is cited as above, but fails to disclose use of a recombinant vector for generation of cells to express a growth factor. The '774 patent is cited for the transformation of stem cells and fibroblasts using vectors with promoters and polyadenylation signals.

As an initial point, applicants challenge the rejection on the grounds that Hadlock, dealing with neural regeneration conduits, has little or no connection with the '774 patent, which is drawn to expression of erythropoietin, a compound that is of no relevance to nerve cells. Thus, there is no logical basis for the examiner's combination of the '774 patent with Hadlock.

[3] Paragraph 7 of the declaration contains information that is not presented in the instant application.

The examiner states that column 13, line 64 reports the transformation of "stem cells and fibroblasts." This is utterly false. There is absolutely no mention of fibroblasts, and the reference teaches ***hematopoietic*** stem cells – clearly a far cry from nerve progenitors. In a valid §103 rejection, the reference must posit their own combination. *In re Vaeck*, 20 U.S.P.Q.2d 1438 (Fed. Cir. 1991). Here, there is no such teaching.

Furthermore, as discussed above, claim 1 has now been amended to recite the elements of previous claim 4, namely, inducible expression of growth factors. Hadlock is silent on this feature. The '744 patent appears to mention, in passing, "inducible" promoters at column 9, line 27. However, as will be explained, there is insufficient motivation in Hadlock or the '744 patent to select truly "inducible" promoters as now claimed.

As explained in the present application, inducibility of a promoter is an important aspect of the invention in that it permits ***regulated*** expression of the growth factors that are needed to stimulate nerve growth, followed by removal of the stimulatory factor by withdrawal of the inducer. Much to the contrary, the '744 patent teaches on that "high level" expression is desired. See, *e.g.*, Column 3, line 38; Column 4, line 48-49; Column 9, line 12; Column 11, lines 1-2 and 52. In addition, it is also suggested that, in the particular situation of transgenic animals, tissue specific expression is desired, and that one way of achieving tissue specific expression is through the use of an inducible promoter. See Column 12, lines 29-67 and Column 12, lines 1-32. Thus, the thrust of the '744 patent is to achieve high expression, possibly in particular tissues, but without concern for any regulation. Thus, the notion of turning transgene expression on and off in a regulated fashion is not contemplated.

In light of these observations, applicants submit that there is no teaching or suggestion in the '774 patent to prepare a device comprising engineered nerve cells expressing, in an inducible

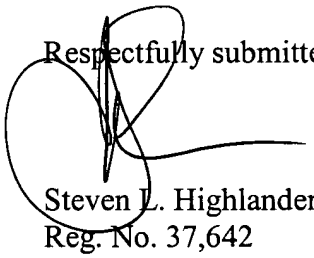
fashion, nerve cell growth factors. To the contrary, the overwhelming balance of the '774 patent is that high level expression is the ***only*** goal. As such, there were be no logical reason to read the '774 patent as teaching the benefits of inducible expression, and as admitted on the record, Hadlock had not even considered such an option, negating a possible suggestion from that reference.

In sum, the examiner has failed to establish that one of skill in the art, reading the citeed references, (a) would have any reason to combine the two cited references or (b) find any motivation to select the engineered nerve cells and conduits of Hadlock and combine them with an inducible promoter as mentioned (in passing) in the '774 patent. As such, there is no basis for alleging *prima facie* obviousness of the claims as presented for reconsideration.

X. Conclusion

In light of the foregoing, applicants respectfully submit that all claims are in condition for allowance, and an early notification to that effect is earnestly solicited. Should the examiner have any questions regarding this response, a telephone call to the undersigned attorney at (512) 536-3184 is invited.

Respectfully submitted,



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CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450, on the date below:

April 28, 2004
Date

Steven L. Highlander

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Gregory R.D. EVANS *et al.*

Serial No.: 09/910,681

Filed: July 20, 2001

For: REGULATED GROWTH FACTOR DELIVERY FOR ENGINEERED PERIPHERAL NERVE

Group Art Unit: 1614

Examiner: Maria Marvich

Atty. Dkt. No.: UTSC:646US/SLH

DECLARATION OF GREGORY R.D. EVANS UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. BOX 1450
Alexandria, VA 22313-01450

I, Gregory R.D. Evans, do declare that:

1. I am a United States citizen residing at 10480 Yosemite Way, Tustin, CA 92782.

2. I am the Gregory R.D. Evans named as an inventor on the above-captioned application.

3. A great deal of research has been performed in support of the present invention, some of which is included in the application as filed, and some of which has been generated subsequent to the filing. Taken together, these data demonstrate the operability of the present invention.

4. Prior to filing of the instant application, early stage experiments used solid (*i.e.*, no lumen) polymer conduits employed poly(L-glycolic acid) (PLGA) foams fabricated by a solvent casting particulate leaching technique using NaCl as the leachable component (Mikos *et al.*, 1994). The foams were assessed for their ability to serve as a biodegradable scaffold for peripheral nerve regeneration in sciatic nerve defects of Sprague Dawley and to evaluate their biocompatibility with regenerating axons. Nerve regeneration was noted around the periphery of the solid biodegradable conduit, but many conduits were fractured leading to new geometric scaffold designs.

Subsequent studies were performed to determine the feasibility of forming tubular structures from these PLGA constructs. *In vivo* studies demonstrated axonal migration within the conduit's lumen, accompanied by vascular in-growth. Several axons demonstrated new myelin formation (Evans *et al.*, 1998a; 1998b). Based on the success of these pilot projects, we chose to evaluate the PLGA engineered conduit for a longer time period. Unlike the previous pilot studies, these conduits were extruded, adding further stability and consistency. These tubular conduits were interposed into a 15 mm right sciatic nerve defect of 20 Sprague Dawley rats and functional evaluation (walking track analysis) was performed monthly. All conduits remained flexible, allowing mobility of the rat extremity without breakage of the conduits. However, partial collapse of the conduits and elongation to 18 mm was noted. Histomorphology demonstrated axonal migration and nerve tissue advancement through the entire conduit and into the distal nerve stump at 12 week. This was the first evidence of distal nerve reinnervation with the use of our fabricated polymer scaffolds (Evans *et al.*, 1998b).

Because of the partial collapse and elongation noted with the use of PLGA conduits over 12 weeks, alternative polymer substrates were examined. Poly(L-lactic acid) (PLLA) (Birmingham Polymers, Birmingham, AL) offers a more stable geometric structure. Consequently, in a similarly designed extrusion process, PLLA was substituted for the *in vivo* trials as previously outlined (Widmer *et al.*, 1998; Evans *et al.*, 1999). PLLA conduits did not demonstrate collapse or elongation. The results for PLLA were significantly improved over those for 75:25 PLGA and suggest that PLLA porous conduits may serve as a scaffold for peripheral nerve regeneration (Evans *et al.*, 1999).

In order to determine the structural integrity of the PLLA and PLGA conduits, mechanical testing was performed (Widmer *et al.*, 1998). Previous work, as outlined above, focused on one component to support nerve regeneration and tissue engineered constructs – the scaffold. Biodegradable polymers have been used for conduit fabrication in an attempt to evaluate this tissue-engineered construct for nerve regeneration, and they serve as a well-established structure to test regulated NGF delivery.

PLLA conduits were further tested in long-term *in vivo* studies (Evans *et al.*, 2000). Thirty-one Sprague Dawley rats were utilized. Throughout all time periods, the PLLA conduit remained structurally intact and demonstrated tissue incorporation and vascularization. There was no evidence of conduit collapse or breakage with limb ambulation. Moreover, there was no evidence of conduit elongation at eight months as previously observed with the 75:25 Poly(DL-lactic-co-gylcolic acid) (PLGA) conduits. The study confirmed our belief that PLLA conduits were a viable scaffold for long-term nerve gap replacement (Evans *et al.*, 2000). These data are presented in Examples 1 and 2 of the instant application.

5. The next effort was directed to finding other nerve components to make the conduits bioactive, specifically, support cells. Schwann cells from the sciatic nerve and brachial plexus of 15 neo-natal Lewis rat pups where harvested. Two Schwann cell concentrations (1×10^4 and 1×10^6/ml) were combined with a collagen matrix (Vitrogen) for implantation into the PLLA conduits. The mean data from walking track analysis (SFI) demonstrated no group differences from autogenous nerve measured over the 4 months. By 4 months, there was no significant difference in gastrocnemius muscle weight in any of the experimental groups when compared to isograft controls. At 4 months, the distal nerve demonstrated a statistically lower number of axons/mm^2 for the 1×10^6 SC/ml, PLLA control and 1×10^4 SC/ml groups. The nerve fiber density was noted to be significantly lower in all of the groups compared to isograft controls by 4 months (Evans *et al.*, 2002).

The purpose for Schwann cell implantation was to deliver NGF by their autogenous production. It was noted, however, that during the study Schwann cells were difficult to expand *in vivo* and maintain. Results with Schwann cells were not significantly different from control conduits. Further, we are unable to regulate the timing or dose of growth factors through this system. Because of these reasons and a need for more controlled NGF delivery, alternative cells were selected. The ability to regulate time and potentially the dose secretion of NGF using these transfected HEK-293 cells is theoretically superior to the above study. Further, HEK-293 cells are technically easier to, expand *in vivo* and maintain.

In an attempt to replace Schwann cells and create a bioactive conduit, we hypothesized that dermal fibroblasts could be genetically modified to act like Schwann cells to deliver required growth factors (*e.g.*, nerve growth factor, NGF). In this experiment, rat dermal fibroblasts (DFBs) were genetically modified to release rat NGF (Patrick *et al.*, 2001). The NGF release

was determined from transfected or control DFBs over a three day period. NGF release from NGF transfected DFBs steadily increased to a value of 111 pg NGF/mL, with 72 hrs results being significantly different than both 24 and 48 hrs ($p \leq 0.002$ and $p \leq 0.01$, respectively). NGF release from NGF transfected DFBs was markedly higher than control DFBs or DFBs transfected with vector alone ($p \leq 0.002$).

To remove the confounding problem of increasing DFB number with increased time, NGF released by transfected DFBs was normalized to cell number. This results in a maximal release of 1.2 pg NGF /mL/10^3 cells from NGF-transfected DFBs at 72 hrs. For all time periods, DFBs transfected with rat NGF expressed markedly higher levels of NGF compared to control DFBs or DFBs transfected with vector alone. Thus, we demonstrated that rat DFBs can be genetically modified to act like Schwann cells to deliver NGF. In addition, we have demonstrated statistically significant differences in NGF released from DFBs transfected with an expression vector encoding rat ß-NGF when compared to control DFBs or DFBs transfected with vector alone. The NGF transfected DFBs demonstrate a maximal NGF release rate during the initial 24 hrs. These data are presented in Example 3 of the instant application.

6. Next, human dermal fibroblasts (hDFBs) were genetically modified to release human NGF using an inducible system. *In vitro* and *in vivo* NGF secretion from transfected hDFBs was quantitatively determined, as well as NGF bioactivity (Patrick *et al.*, 2001). Untransfected hDFBs served as a negative control in all *in vivo* and *in vitro* experiments. Muristerone A (3 μM) was used as the inducing agent for all *in vitro* and *in vivo* experiments. The *in vitro* NGF release was determined from transfected hDFBs with (MurA (+)) and without (MurA (-)) the inducing agent Muristerone A over a three-day period. Knowing the volume of culture media

the results were converted to a release rate curve. MurA (+) hDFBs demonstrated maximal NGF release at day 1 (5.1 ± 0.2 ng NGF/10^6 cells/day), followed by a markedly lower, sustained release rate at days 2 and 3 (2.4 ± 0.2 ng NGF/10^6 cells/day and 2.8 ± 0.1 ng NGF/10^6 cells/day, respectively).

In vivo experiments were conducted in nude rats. After shaving the back, a longitudinal incision (~2 cm each) was made through the skin of the right and left flank. Individual "pockets" for each collection chamber were prepared in the subcutaneous space of both flanks by blunt dissection. Collection chambers were inserted into each pocket and capped by suturing muscle to each end with 4-0 Prolene suture (Ethicon). The sutured muscle caps provided both a tight seal for the collection chamber and a vascular supply to maintain the viability of injected hDFBs. The sealed collection chambers were filled with either transfected hDFBs with Muristerone A (TFB/MurA (+)), transfected hDFBs without Muristerone A (TFB/MurA (-)), untransfected hDFBs (NTFB), or phosphate buffered saline (PBS) using a 1 cc tuberculin syringe with 27 G needle (Becton Dickinson). All transfected and untransfected hDFBs were injected at 10^6 cells/mL. The collection chambers were left *in vivo* one and two days. After the elapsed time, the rats were euthanized with CO_2 and the fluid within the collection chamber withdrawn with a 1 cc tuberculin syringe with a 27 G needle (Becton Dickinson) and placed in a Eppendorf tube for subsequent NGF ELISA.

At both 1 and 2 days, TFB/MurA (+) possessed significantly higher NGF levels (2,074 ± 257 *versus* 1620 ± 132, respectively) when compared to negative controls ($p \leq 0.05$ and $p \leq 0.003$, respectively). There was no statistical difference between the TFB/MurA (-), NTFB, and PBS groups at either day in keeping with the hypothesis that these three groups should yield

wound fluid NGF levels. In agreement with the release kinetic trends, the NGF released upon inducing transfected hDFBs was greatest at day 1 and decreased by 22% at day 2.

Thus, we have demonstrated that hDFBs can be genetically modified and induced to act like Schwann cells to deliver NGF *in vitro* and *in vivo*. Further, Liu *et al.* (1995) previously quantified the amount of NGF in the wound fluid around a transected nerve. They reported NGF concentrations ranging from 310 ± 60 pg NGF/mL to 950 ± 140 pg NGF/mL over a 7 day period. Serum levels of NGF over the same time period ranged from 30 ± 8 pg NGF/mL to 50 ± 5 pg NGF/mL. Our *in vitro* NGF concentrations ranged from 71.2 pg NGF/mL at 24 hrs to 111 pg NGF/mL at 72 hrs. Hence, our released concentrations are within the same order of magnitude as wound fluid concentrations and are greater than reported serum levels. When data are converted to release rates, we obtain a maximal release rate of 1.2 ng NGF/10^6 cells/day. Thus, if we placed 10^6 transfected DFBs within a nerve conduit, we would have an order of magnitude more NGF than measured in transected nerve wound fluid given equal volumes. These data are presented in Example 4 of the instant application.

7. Though we demonstrated that human dermal fibroblasts (hDFBs) can be genetically modified to secrete NGF, production in these cells was noted to be transient and limited to a three day period. Collagen deposition leading to scar formation raised further concerns regarding the use of hDFBs for NGF release and prompted the search for different sources of growth factor delivery. In this experiment, Human Embryonic Kidney cells (HEK-293) were genetically modified to release human NGF using an inducible system referred to as hNGF-EcR-293 cells. Advantages of HEK-293 cells are that they are a human cell line, which is readily transfected and commercially available. Additionally, lack of collagen secretion invokes less

possibility of scar formation than hDFB's. Inducible expression of NGF allows increased control over the time interval and magnitude of release and can be used to prevent excessive growth factor production leading to potentially detrimental outcomes.

NGF secretion from transfected HEK-293 cells was quantitatively measured *in vitro* over a 9-day period following incrementally increased concentrations of the inducing agent Ponasterone A. Additionally, *in vitro* bioactivity of secreted NGF was assessed over the 9-day period using a PC-12 cell differentiation assay. Ponasterone A is an analog of Muristerone and was selected in these current experiments. HEK-293 cells were obtained from Invitrogen and were cultured according to the manufacturer's instructions. PC-12 cells were cultured as previously described (Raffioni *et al.*, 1998). The expression vector pIND-hNGF and pVgRXR (regulator vector) were transfected into HEK-293 cells using LIPOFECTAMINE™ 2000 (Invitrogen) per the manufacturer's instructions. Selection of cells containing stable integration of the plasmid DNA was performed by adding Zeocin (200 μg/mL) and G418 (600 μg/mL) to the medium. Individual clones were selected by serial dilutions. The antibiotics in the medium for culturing individual clones were subsequently reduced to 200 μg/mL Zeocin and 200 μg/mL G418. The plasmid pIND-NGF was obtained from our previous dermal fibroblast study following amplification of human NGF cDNA, digestion, and insertion as previously described in the above study (Patrick *et al.*, 2001). Correct insertion was confirmed by restriction analysis and sequencing as previously described (Patrick *et al.*, 2001). hNGF-EcR-293 cells were left untreated or treated with DMEM containing 2 μM Ponasterone A for 1, 3, 5, 7 or 9 days without changing the medium. Five hundred μL aliquots of medium from each timepoint were analyzed for the presence of NGF using an ELISA according to the manufacturer's instructions (Promega). All experiments were performed in duplicate or triplicate. Parallel samples were

used to determine the total number of cells in each treatment condition, and the amount of NGF was determined relative to the number of cells present at each time point.

PC-12 cells were used to assess bioactivity of secreted NGF with PC-12 differentiation being the measured end-point. PC-12 cells have been shown to dose-dependently change their phenotype and extend neurite-like processes in response to bioactive NGF (Sofroniew *et al.*, 2001). Differentiation was defined as 2 or more neurites extending at least one cell body in length. Briefly, hNGF-EcR-293 cells were seeded 5 x 10^4 cells per well of a 12-well plate and were left untreated or exposed to various concentrations of Ponasterone A for the times indicated. On the day prior to the removal of the aliquot to be tested, 1000 PC-12 cells were seeded into each well of a poly-L-lysine-coated 12-well plate. For each sample to be analyzed, 500 μL was transferred from the hNGF-EcR-293 cell wells to a 1.5 mL microcentrifuge tube and subjected to microcentrifugation at 14,000 x g for 10 min to separate any cells from the medium. Four hundred μL of the cleared medium was then transferred into a single well containing PC-12 cells. PC-12 cells were also left in their normal culture medium or medium supplemented with purified 50 ng/mL of 2S NGF (Harlan Bioproducts) as negative and positive controls, respectively. After 48 hrs of exposure of PC-12 cells to NGF or medium from hNGF-EcR-293 cells the neurite-bearing cells were quantitated by determining the percentage of cells possessing two or more neurites of at least two cell body widths.

NGF release was determined from hNGF-EcR-293 cells with [PonA(+)] and without [PonA(-)] the inducing agent Ponasterone-A over a 9-day period. Because PonA(+) hNGF-EcR-293 cells expressed markedly greater levels of NGF compared to PonA(-) cells, values from non-induced samples were normalized to zero and values from induced samples were adjusted accordingly. To remove the potential confounding problem of increasing cell number with

increased culture time, cells were counted on each day and NGF released by hNGF-EcR-293 cells was also normalized to cell number. NGF production steadily increased over a 9-day period from 126.4 $\pm$ 4.3 pg/mL to a maximal release of 9.1 $\pm$ 2.6 ng/mL at day 9. Maximal NGF production per cell was 8.5 $\pm$ 2.5 pg/ 10^3 cells at day 9. NGF release from [Pon A+] hNGF-EcR-293 cells was not statistically significant between different concentrations of Ponasterone A (1 μm, 2 μm, and 3 μm) at days 1, 5 and 9. NGF release between the varying doses of Ponasterone A was, however, noted to be statistically significant at days 3 and 7 ($p \leq 0.05$). PC-12 cells exposed to cell media from induced hNGF-EcR-HEK-293 cells demonstrated markedly higher levels of differentiation compared to PC-12 cells exposed to both media from non-induced hNGF-EcR-293 cells and cell media alone (DMEM) at days 3 and 5. Peak differentiation was noted at day 5 with 100% (per 100 cells counted) of PC-12 cells differentiated at all three concentrations of Ponasterone A.

Thus, our results have demonstrated that HEK-293 cells can be genetically engineered *in vitro* to secrete increasing levels of bioactive NGF over a nine-day period with the use of an inducible expression vector encoding human NGF when compared to non-induced control hNGF-EcR-293 cells. Bioactivity of secreted NGF was noted to be maximal 5 days following addition of Ponasterone A. Measurement of secreted NGF peaked at day nine with a maximum expression of 9.1 $\pm$ 2.6 ng/mL (Jimenez & Evans, 2002, unpublished data).

8. Based on the foregoing research results, I believe that we have now proven that the invention, as presently claimed, will work. Thus, although we have not yet completed the final stages of our research, the evidence presented here indicates that using biocompatible conduits and engineered cells as disclosed, one can deliver nerve cells and support their growth *in vivo*.

9. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

Date April 26 2004

Gregory R.D. Evans